Control of insect vectors and plant viruses in protected crops by novel pyrethroid-treated nets

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Abstract

BACKGROUND: Long-lasting insecticide-treated nets (LLITNs) constitute a novel alternative that combines physical and chemical tactics to prevent insect access and the spread of insect-transmitted plant viruses in protected enclosures. This approach is based on a slow-release insecticide-treated net with large hole sizes that allow improved ventilation of greenhouses. The efficacy of a wide range of LLITNs was tested under laboratory conditions against *Myzus persicae*, *Aphis gossypii* and *Bemisia tabaci*. Two nets were selected for field tests under a high insect infestation pressure in the presence of plants infected with *Cucumber mosaic virus* and *Cucurbit aphid-borne yellows virus*. The efficacy of *Aphidius colemani*, a parasitoid commonly used for biological control of aphids, was studied in parallel field experiments.

RESULTS: LLITNs produced high mortality of aphids, although their efficacy decreased over time with sun exposure. Certain nets excluded whiteflies under laboratory conditions; however, they failed in the field. Nets effectively blocked the invasion of aphids and reduced the incidence of viruses in the field. The parasitoid *A. colemani* was compatible with LLITNs.

CONCLUSION: LLITNs of appropriate mesh size can become a very valuable tool in combination with biocontrol agents for additional protection against insect vectors of plant viruses under IPM programmes.

Keywords: Treated nets; aphids; whiteflies; parasitoids; virus control; SADIE

1 INTRODUCTION

Vegetable crops suffer from economically damaging insect pests and insect-transmitted virus pathogens. Integrated pest management (IPM) programmes entail an interdisciplinary combination of chemical and biological measures to manage pest damage.[1] The control of these pests generally involves intensive insecticide spraying, with undesirable effects on the environment, growers and public health. Therefore, there is an urgent need to develop alternatives under the scope of IPM. The use of physical barriers is an excellent method to reduce pest access to crops and impede disease transmission to plants.[2] The selection of an appropriate insect screen depends on several factors: the thoracic size of the insect, the size and geometry of the hole and the way threads are interlaced. Unfortunately, effective barriers against small insects also reduce the airflow and the ventilation inside greenhouses, which frequently increases fungal problems.[3,4] For this reason, new strategies together with physical barriers need to be developed to prevent damage due to small insect pests and reduce the incidence of plant pathogens.

Insecticide-treated nets were developed long ago as bednets in public health to give protection against malaria.[5] This strategy was approved for use with pyrethroids, compounds that exhibit a rapid knockdown effect and high insecticidal potency at low dosage without mutagenic or teratogenic effects.[6] The insecticide may be applied to the net surface by immersion or spraying, but also by incorporation in the process of making the yarns in the factory. In the latter case, the nets are called long-lasting insecticide-treated nets (LLITN), and the insecticide may persist more than 3 years under field conditions.[7]

Field experiments using LLITNs have demonstrated promising results against agricultural pests such as mites in African eggplant, resulting in higher yields,[8] and in brassica crops.[9–11] These nets are cost effective in cabbage production. LLITNs serve as an effective barrier to control a wide range of lepidopteran pests, including the diamondback moth *Plutella xylostella* L. (Lepidoptera: Plutellidae) and *Hellula undalis* (Fabricius) (Lepidoptera: Crambidae), or the aphid *Lipaphis erysimi* Kaltenbach (Hemiptera: Aphididae), but not

against the cabbage whitefly *Aleyrodes proletella* L. (Hemiptera: Aleyrodidae).[9–11]

Nevertheless, more accurate studies under laboratory and field conditions are necessary to understand fully the mechanism of action of this novel pest control strategy and its compatibility with natural enemies commonly used in biocontrol under protected enclosures. To date, little attention has been paid to this issue, and there are no studies concerning the effects of LLITNs on the behaviour or performance of natural enemies. The bioassays in this study were designed to select the most appropriate LLITN among nets with different properties (insecticide compounds, dosages and hole size) against three key pests in vegetable crops: *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), *Myzus persicae* Sulzer and *Aphis gossypii* Glover (Hemiptera: Aphididae). These insects are polyphagous and cause great concern because of direct damage by extracting plant fluids, but more importantly because of their ability to transmit devastating virus pathogens.[12,13] Standard insecticide applications have led to the development of insect resistance to many substances,[14] so that the integration and understanding of control alternatives is necessary.[15,16]

Aphids are the most important vectors of plant viruses.[17] Therefore, it is crucial to interfere with the immigration and dispersal of potentially viruliferous vectors inside protected crops. We have studied two major aphid-transmitted plant viruses affecting cucurbits: *Cucumber mosaic virus* (CMV, *Cucumovirus*) and *Cucumber aphid-borne yellows virus* (CABYV, *Polerovirus*). The two viruses have different transmission modes. CMV is transmitted in a stylet-borne, non-persistent manner during brief probes on epidermal cells, while CABYV is a circulative, non-propagative phloem-restricted virus that requires long feeding probes for transmission.

The objective of our work was to test under laboratory and field conditions a wide range of LLITNs designed to reduce the incidence of pests and viruses of horticultural crops grown under protected enclosures (greenhouses or net-houses). The new approach is based on a slow-release insecticide-treated net that can provide relatively larger mesh sizes and thereby greater airflow than standard untreated nets. Moreover, the effect of such nets on the spatial distribution of aphids and aphid-transmitted viruses was studied using spatial analysis by distance indices (SADIE) methodology.[18–21] Finally, the compatibility of LLITNs with the aphid parasitoid *Aphidius colemani* Viereck (Hymenoptera: Aphidiinae) was tested under field conditions.

Table 1. Characteristics of the nets used in the glass vial experiments

Net code	Colour	Hole (mm^2)	UV-add[a]	Deltamethrin (g kg^{-1} net)
151	Green	2.41	No	–
210	White	1.93	No	–
C25warp	White	0.73	No	–
149	White	3.42	No	2.0
412	White	3.38	No	2.0
404	Light blue	2.78	No	1.4
406	Green	2.77	Yes	1.4
150	Blue	2.65	No	2.0
190	Dark blue	2.62	No	2.0
147	Yellow	2.59	No	4.0
405	White	2.47	Yes	1.4
191	Light blue	2.07	No	2.0
206	Yellow	2.06	No	4.0
148	White	2.00	No	4.0
207	White	1.82	No	4.0
25	White	0.66	No	1.2
25-30	White	0.35	No	2.8
Net code	Colour	Hole (mm^2)	UV-add	Bifenthrin (g kg^{-1} net)
1.4	Yellow	0.77	No	–
C7 × 11	Yellow	0.70	No	–
2.4	Yellow	0.56	No	–
3.4	Yellow	0.45	No	–
C10 × 11	Yellow	0.41	No	–
64/11/07	Yellow	0.29	No	–
42	Yellow	0.12	No	–
1	Yellow	0.83	Yes	4.0
TR11-291	Yellow	0.71	Yes	4.0
2	Yellow	0.60	Yes	4.0
TR11-290	Yellow	0.46	Yes	3.8
3	Yellow	0.44	Yes	5.0
64/11/08	Yellow	0.29	Yes	2.1
40	Yellow	0.12	Yes	3.4
Net code	Colour	Hole (mm^2)	UV-add	Chemical compound
196	Violet	2.92	No	Deltamethrin + PBO[b]
195	Pink	2.67	No	PBO

[a] Addition of UV-blockers.
[b] Piperonyl butoxide.

2 MATERIALS AND METHODS

2.1 Long-lasting insecticide-treated nets

Nets were made of polyethylene yarns knitted in different patterns and provided by the companies Intelligent Insect Control SAS (Castelnau Le Lez, France) and Ginegar Plastic Products Ltd (Kibbutz Ginegar, Israel). The net yarns were pretreated with insecticides during the manufacturing process to produce LLITNs. Yarn diameter ranged from 0.13 to 0.29 mm. A total of 23 insecticide-treated nets and ten untreated controls classified according to the following criteria were tested: a hole size ranging from 0.12 to 3.42 mm^2, different UV-blocking additives and various insecticide compounds and dosages (Table 1).

2.2 Insect cultures and virus isolates

A clonal population of *Myzus persicae* and *Aphis gossypii* was started from a single virginoparous apterae collected in Madrid and Almería (Spain) respectively. Both aphid colonies were maintained in a growth chamber at a photoperiod of 16:8 h (light:dark), 23:18 °C (day:night) and 60–80% RH. Winged *M. persicae* and *A. gossypii* aphids were produced by rearing aphids at high densities on turnip (*Brassica rapa* L. cv. 'Just Right') (Takii & Co. Ltd, Kyoto, Japan) and melon plants (*Cucumis melo* L. cv. 'Primal') (Syngenta Seeds B.V., Enkhuizen, The Netherlands) respectively. Whitefly *Bemisia tabaci* Q biotype donated by La Mayora Experimental Station (Málaga, Spain) was reared on melon plants in greenhouse facilities at a photoperiod of 16:8 h (light:dark), 20–23 °C and 70–80% RH. Identity of biotype status of the population in rearing was periodically confirmed by determining the sequence of cytochrome oxidase I mitochondrial gene.[22]

Cucumber (*Cucumis sativus* cv. 'Marumba') (Enza Zaden S.L., Almería, Spain) plants were inoculated with *Cucumber mosaic virus* (CMV, *Cucumovirus*) and *Cucumber aphid-borne yellows virus* (CABYV, *Polerovirus*) 13 days after sowing at the one-true-leaf stage

and used 5 weeks post-inoculation as viral sources. Plants were mechanically inoculated with CMV isolate M6, obtained from a melon crop in 1996 in Tarragona (Spain) and kindly provided by Dr E Moriones (EELM-CSIC, Spain). CMV-infected plants were maintained at ICA-CSIC in an insect-proof chamber at a photoperiod of 16:8 h (light:dark), 25:20 °C (day:night) and 90% RH. The CABYV isolate was kindly provided by Dr H Lecoq (INRA, France) and obtained from zucchini squash in 2003 in Montfavet (France). It was inoculated to cucumber plants (cv. 'Marumba') by aphid serial transmission. *A. gossypii* adult aphids were put on CABYV-infected source plants during 48 h, and nymphs produced during this period were allowed to feed for one extra day, reaching an acquisition access period (AAP) of 3 days. After the AAP, 25 nymphs grown on the CABYV-infected plants were transferred to each healthy receptor plant for an inoculation access period (IAP) of 3 days. Then, nymphs were manually removed. CABYV-infected plants were maintained in a chamber at a photoperiod of 16:8 h (light:dark), 20:16 °C (day:night) and 60% RH.

2.3 Laboratory assays

Experiments were conducted at the Institute of Agricultural Sciences (ICA) of the Spanish National Research Council (CSIC, Madrid, Spain). A novel experimental set-up was designed to force insects to go through the insecticide-treated net so that the effectiveness of the net killing the insects could be evaluated (Fig. 1a). All assays were conducted using an experimental tube composed of two glass cylinders (12 cm long × 4 cm in diameter) separated by the test net. An untreated net of the same mesh and vials with no net were used as controls in every experiment. Insects were released in the bottom chamber, and a leaf of a preferred aphid host plant was placed in the top chamber as target. The vial was surrounded with black cardboard except at the top, which was covered with a thin cloth that allowed ventilation and light penetration into the structure. Both the target leaf and light coming from the top were the stimuli to induce insects to climb up and cross the LLITN. Insects located either feeding on the target leaf, dead or alive in the release chamber were assessed 6 and 24 h after aphid and whitefly release respectively. Mortality values were corrected using Abbott's formula.[23] Trials were conducted in the laboratory at room temperature (22–24 °C).

The influence of deltamethrin concentration on *M. persicae* mortality was tested with different insecticide concentrations ($n = 9$), presence of UV-blocking additives ($n = 3$), net colour (white versus yellow) ($n = 6$) and chemical compounds [deltamethrin, piperonyl butoxide (PBO) and a combination of both] ($n = 6$), using a turnip leaf as target. The efficacy of deltamethrin-treated nets against *B. tabaci* ($n = 3$) was tested using a tomato leaf as target (*Solanum lycopersicum* L. cv. 'Marmande') (Semillas Fito S.A., Barcelona, Spain). Ten *M. persicae* and 50 *B. tabaci* were released in the bottom chamber for the deltamethrin trials.

In parallel experiments, net samples were placed in trays and exposed for 1 month to field conditions at La Poveda Experimental Farm (Arganda del Rey, Madrid, Spain) (40° 18′ 58″ N, 3° 29′ 05″ W) during winter and spring seasons to assess the persistence of delthametrin ($n = 4$) in the nets. Daily average climatic data were 6.4 °C mean temperature, 11.7 °C maximum temperature, 1.8 °C minimum temperature and 6.5 MJ m^{-2} day^{-1} radiation during winter, and 15.8 °C mean temperature, 23.4 °C maximum temperature, 8.9 °C minimum temperature and 21.1 MJ m^{-2} day^{-1} radiation during spring.

Bifenthrin was also tested because it has been reported to have a longer persistence and stability than deltamethrin.[24] Therefore, a similar experimental procedure was applied to compare different types of bifenthrin-treated net ($n = 9$) and the persistence of bifenthrin in nets exposed to field conditions 2 months during the autumn season at the field experimental site (Arganda del Rey, Madrid) ($n = 4$). These nets were also tested in glass tube tests against *A. gossypii* using a cucumber leaf (*C. sativus* L. cv. 'Ashley') (Rocalba S.A., Gerona, Spain) and against *B. tabaci* with a tomato leaf (cv. 'Marmande') as targets. Twenty *A. gossypii* and 20 *B. tabaci* individuals were tested in each vial for the bifenthrin assays.

2.4 Determination of the insecticide concentration of LLITNs

Insecticide analysis was done using an adaptation of the CIPAC method 454/LN/M/3.2 for alpha-cypermethrin by gas chromatography with flame ionisation detection (GC-FID).[25] Insecticide was determined by extraction in xylene (25 mL) of net samples (200 mg) in a conical flask. The flask was connected to a condenser and heated to reflux for 60 min until the net sample was completely dissolved. The solution was cooled to room temperature,

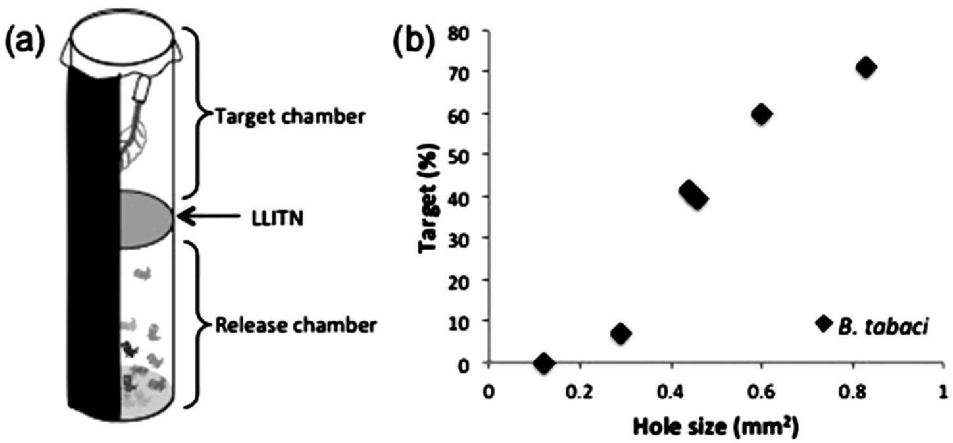

Figure 1. (a) Schematic diagram of the laboratory experimental set-up. Half of the drawing is shown without the black cardboard covering the tube, to show the inner structure. (b) Mean percentage of *Bemisia tabaci* feeding on the target after crossing a range of bifenthrin-treated nets with different hole sizes (net 40, 0.12 mm^2 hole size; net 64/11/08, 0.29 mm^2 hole size; net 3, 0.44 mm^2 hole size; net TR11-290, 0.46 mm^2 hole size; net 2, 0.60 mm^2 hole size; net 1, 0.83 mm^2 hole size).

filtered through a nylon filter (0.2 μm) and analysed by GC-FID with a system comprising an Agilent Technologies 7890A gas chromatograph, an Agilent Technologies 7693A autosampler and a capillary fused silica column (5% phenyl methyl siloxane 0.25 μm, 30 m × 0.25 mm) (Agilent Technologies Inc., Santa Clara, CA) with an injection volume of 1 μL and a flow rate of 300 mL min^{-1}. Insecticide content was calculated with an external standard calibration.

2.5 Efficacy of LLITNs in field conditions against aphids and whiteflies

Field trials were designed to test the incidence of pest population dynamics and plant viruses in natural conditions. The two nets that provided the best results in the laboratory trials and could be produced on a large scale were tested in two field experiments at La Poveda Experimental Farm during autumn of 2011 and 2013. Three identical tunnel-type net-houses (8 m long × 6.5 m wide × 2.6 m high), 5 m apart and with the same E–W orientation, soil properties and irrigation regimes, were used. Each net-house was divided into two equal experimental plots separated with a vertical net following a split-plot design with three replicates. Every plot held 42 cucumber (*C. sativus* var. 'Marumba') plants, distributed in seven rows. An experimental net section (3.5 m long × 4.3 m wide) was placed on each side of the tunnels, replacing the standard transparent polyester netting that covered the entire net-house. During 2011, the net-house contained a yellow test net of equal mesh (net TR11-290, 0.46 mm^2 hole size) either treated with 3.8 g bifenthrin kg^{-1} or untreated. In 2013, according to the results of the first field study and after a second set of laboratory trials, the new net 64/11/08 was tested. The hole size was reduced to 0.29 mm^2, either treated with 2.1 g bifenthrin kg^{-1} net or untreated, with the aim of decreasing the numbers of living *B. tabaci* crossing the net. On the outer sides of the net-houses, an additional row of six cucumber plants (two infected with CMV, two infected with CABYV and two non-infected) was transplanted to provide inoculum sources at both sides of the net-house. In 2011, 2 days after transplant, 240 winged *A. gossypii* and 500 *B. tabaci* per plot were released on the virus-infected cucumber plants that were transplanted on the outer side of the net-house. Aphids were directly released on the leaf surface to improve settlement and viral acquisition. Whiteflies were released at the canopy level along the virus-infected source plants. In 2013, 360 winged *A. gossypii* and 400 *B. tabaci* per experimental plot were released. Aphids and whiteflies crossing the nets were assessed by counting their absence/presence in the cucumber plants inside the experimental plots. Furthermore, aphid density in 11 marked cucumber plants inside each experimental plot and in the virus source plants on the outer sides was monitored weekly by using the following scale previously used in similar studies:[26] [0 (0 aphids), 1 (1–4 aphids), 2 (5–19 aphids), 3 (20–49 aphids), 4 (50–149 aphids), 5 (>150 aphids)]. Leaf samples from each cucumber plant under experimental plots were collected 9 weeks after insect release, and virus infection was confirmed by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using specific commercial antibodies against CMV (Agdia Inc., Elkhart, IN) and CABYV (Sediag S.A.S., Longvic, France).[27] Net samples were also collected at different time intervals during each field experiment to measure the remaining insecticide concentration after field exposure. Daily average climatic conditions were 15.2 °C temperature, 61.2% RH, 12.3 MJ m^{-2} day^{-1} radiation and 1.5 mm rainfall during 2011, and 16.1 °C temperature, 69.32% RH, 13.3 MJ m^{-2} day^{-1} radiation and 1.0 mm rainfall during 2013.

2.6 Effect of bifenthrin-treated nets on the aphid parasitoid *Aphidius colemani*

The impact of the TR11-290 net on *A. colemani* was tested in a separate and independent set of three tunnels with the same dimensions as those previously described and located on La Poveda Experimental Farm during the autumn season. We followed a similar split-plot design with three replicates, each net-house being divided into two equal experimental plots. Each plot contained a section of either bifenthrin-treated or untreated net (0.46 mm^2 hole size) on each side of the tunnel. Each plot held 42 cucumber plants, distributed in seven rows. At the two-true-leaf stage, three adults of *A. gossypii* were released on each of 11 marked plants inside each experimental plot. As opposed to the field experiments where aphids and whiteflies were released on the outer side of the net-house, the parasitoids (APHIcontrol®; Agrobio, La Mojonera, Spain) were released inside the net-house and hung on a platform placed in the centre of each plot at a rate of 10 adults m^{-2} at 2 weeks after aphid infestation. Insect sampling was performed weekly for 6 weeks by scouting their absence/presence in all plants and by counting their number in the 11 marked plants. Daily average climatic conditions were 15.7 °C temperature, 70.3% RH, 12.9 MJ m^{-2} day^{-1} radiation and 2.3 mm rainfall.

2.7 Spatial analysis

The spatial distribution of aphids and virus spread was studied using the SADIE methodology applied to the data of year 2011. This analysis is based on the distance to regularity, D, the minimum value of the total distance that individuals have to move so that the population is distributed as regularly as possible, and the distance to crowding, C, the minimum value of the total distance that individuals must move to be as aggregated as possible.[18,19] The spatial pattern of data was described by the index of aggregation, *Ia*, the positive patch cluster index, *vi*, and the negative gap cluster index, *vj*.[20] *Ia* denotes the pattern of the population, which by convention is an aggregated sample if $Ia > 1$, a spatially random sample if $Ia = 1$ and a regular sample if $Ia < 1$. For units within patches of relatively large counts close to one another, the patch cluster index would be large. Conversely, the gap cluster index tends to be large in units within gaps of small counts close to one another. Both indices visually indicate the location and extent of cluster in the data, so their values could be contoured with a contouring modelling program such as Golden Surfer, which allows the graphical representation of patches and gaps.[28] Moreover, the degree of local association between aphid presence and virus incidence was calculated with the index of spatial association, X, and contoured as well.[21] In our study, a value of 1 was assigned to plants infested by aphids or infected by virus and a value of 0 to uninfested or non-infected plants for each of the 42 cucumber plants inside each plot.

2.8 Statistical analysis

Data were previously transformed with $\sqrt{x + 0.5}$, x^2, $\ln(x+1)$ or $2 * \arcsin\sqrt{x}$ if needed to reduce heteroscedasticity and achieve normality. Differences among nets in the percentage of mortality and insects feeding on the target leaf in the laboratory assays and in the insect density in field experiments were assessed by a parametric one-way ANOVA test followed by pairwise comparison for least significant differences (LSD) or a Student t-test ($P \leq 0.05$) using IBM Statistics SPSS 21.0 software.[29] When the data did not follow the ANOVA assumptions, a non-parametric Kruskal–Wallis H-test or Mann–Whitney U-test ($P \leq 0.05$) was performed. Insect occupancy

Table 2. Percentage of aphids feeding on the leaf target (%) in laboratory trials, showing the parameters evaluated and values, the nets used in each experiment and the statistics according to a one-way ANOVA test ($P \leq 0.05$)

Insecticide	Parameter evaluated	Net code	Parameter value	Insects on target (%)			F	P
				X	SE			
Deltamethrin	Dose (g kg^{-1} net)	151	–	46.7	9.0	a	22.30	<0.01
		150	2	5.6	1.8	b		
		148	4	1.1	1.1	b		
	UV-additives	151	–	64.2	7.2	a	21.50	<0.01
		406	Yes	14.0	7.2	b		
		405	Yes	7.4	7.4	b		
		404	No	0	0	b		
	Compound	151	–	64.0	5.9	a	34.59	<0.01
		195	PBO	46.3	5.7	b		
		190	Deltamethrin	6.8	1.9	c		
		196	Deltamethrin + PBO	6.7	4.2	c		
	Persistence (g kg^{-1} net)	404	1.4 (unexposed)	0.0	0.0	c	14.12	<0.01
			Winter exposure	25.6	5.0	b		
			Spring exposure	55.3	10.1	a		
		406	1.4 (unexposed)	14.0	7.2		1.66	0.25
			Winter exposure	34.0	1.9			
			Spring exposure	33.0	11.7			
		412	2 (unexposed)	7.4	7.4	ab	5.79	0.03
			Winter exposure	1.3	1.3	b		
			Spring exposure	20.0	3.6	a		
Bifenthrin	Hole size (mm^2)	1	0.83	15.4	6.0	a	17.85	<0.01
		2	0.60	5.2	1.9	ab		
		TR11-290	0.46	1.0	0.7	b		
		TR11-291	0.71	0.0	0.0	b		
		3	0.44	0.0	0.0	b		
	Persistence (g kg^{-1} net)	C10 × 11	–	38.5	8.4	a	17.06	<0.01
		TR11-290	3.8 (unexposed)	1.1	1.1	b		
		TR11-290	3.4 (exposed for 1 month)	1.1	1.1	b		
		TR11-290	3.1 (exposed for 2 months)	38.6	13.7	a		

rate and virus incidence were compared by a χ^2 goodness-of-fit test ($P \leq 0.05$) to check whether the observed frequency distribution was related to the expected frequency distribution using Statview 4.01 software.[30]

3 RESULTS

3.1 Efficacy of LLITNs against aphids in laboratory trials

All LLITNs impregnated with pyrethroids produced high mortality in *M. persicae* and thereby reduced the chances that insects would reach the target. Almost half the aphids reached the target chamber in untreated net 151 and differed significantly from deltamethrin tubes, with fewer insects reaching the target under high concentration (Table 2). The addition of UV-blockers to net 404 did not make any difference with respect to the percentage of aphids reaching the target, although it was significantly higher in untreated net 151 compared with LLITNs 404, 405 and 406 (Table 2). No synergistic effect of the insecticides deltamethrin and PBO was detected. In contrast, PBO alone caused a significantly higher number of *M. persicae* on the leaf when compared with deltamethrin and deltamethrin + PBO nets. PBO-treated net 195 had lower values than untreated net 151 (Table 2). The efficacy of deltamethrin-treated nets decreased over time with sun exposure in two of the three nets tested, 404 and 412 (Table 2). In both cases, the spring-exposed nets significantly increased the percentage of *M. persicae* in the target leaf, and even after winter exposure for net 404. Net 406 was not affected by sun exposure (Table 2). Moreover, no significant differences in mortality were found between net colours white and yellow ($P = 0.078$).

All the bifenthrin LLITNs tested reduced *A. gossypii* presence on the target. The five treated nets statistically differed in the number of aphids reaching the plant target, and the two most promising nets had a hole size of 0.71 and 0.44 mm^2 (Table 2). The numbers of aphids reaching the leaf significantly differed among periods of field exposure. The unexposed and one-month-field-exposed nets demonstrated relatively good performance, with similar values for aphids reaching the target. However, when the net was exposed for 2 months, the percentage of aphids in the leaf increased up to values similar to those of vials with untreated nets (Table 2).

3.2 Efficacy of LLITNs against whiteflies in laboratory trials

Over 80% of the whiteflies tested on deltamethrin-treated nets were alive in the target chamber 24 h after release (Table 3). *B. tabaci* mortality was found to be low in this experiment, showing a minimum of 0.1 ± 0.4% in net 206, increasing up to 17.1 ± 4.6% in net 25. However, significant differences in the percentage of whiteflies reaching the target were found between bifenthrin-treated nets 1, 2 and 3 and untreated nets 1.4, 2.4 and 3.4 (Table 3). Among the treated nets tested during this first laboratory survey, net 3 had

Table 3. Percentage of whiteflies feeding on the leaf target (%) in laboratory trials, showing the parameters evaluated and values, the nets used in each experiment and the statistics according to a one-way ANOVA test ($P \leq 0.05$)

Insecticide	Parameter evaluated	Net code	Parameter value	Insects on target (%)			F	P
				X	SE			
Deltamethrin	Hole size (mm^2)	151	2.41	82.3	1.7		0.46	0.91
		147	2.59	82.4	4.8			
		149	3.42	85.1	2.4			
		150	2.65	84.2	3.7			
		190	2.62	80.9	5.1			
		191	2.07	81.4	3.0			
		148	2.00	83.8	3.3			
		206	2.06	83.7	3.8			
		207	1.82	86.7	4.7			
		25-30	0.35	78.2	5.4			
		25	0.66	72.4	5.4			
Bifenthrin	Hole size (mm^2)	1.4	0.77	90.9	2.2	a	17.80	<0.01
		2.4	0.56	83.1	4.2	a		
		3.4	0.45	83.7	4.7	a		
		1	0.83	71.2	3.5	b		
		3	0.44	41.5	4.8	c		
		2	0.60	59.9	5.3	b		
	Persistence (g kg net)	C10 × 11	–	94.8	1.0	a	19.29	<0.01
		TR11-290	3.8 (unexposed)	39.2	0.8	b		
		TR11-290	3.4 (exposed for 1 month)	84.1	5.9	a		
		TR11-290	3.1 (exposed for 2 months)	92.7	1.8	a		

the lowest value (Table 3). Sun exposure of the bifenthrin-treated net used in the first field experiment caused a significant reduction in efficiency against whiteflies under laboratory conditions from 1 month onwards (Table 3).

After these trials, and according to the results obtained during the first field experiment, we undertook a second set of laboratory assays testing two new LLITNs with a smaller hole size (nets 64/11/08 and 40) (Table 1). Figure 1b shows the relation between hole size and *B. tabaci* reaching the target in a wide range of nets tested during several years. Pore sizes above 0.44 mm^2 were not sufficient to prevent living whiteflies feeding on the leaf. Net 40 was too dense (0.12 mm^2 hole size) and provided a physical control, as no whiteflies were found on the leaf in the untreated vials with standard net 42. However, LLITN 64/11/08 with a 0.29 mm^2 pore size gave promising results, as only 6.77 ± 2.46% whiteflies reached the target, and was therefore selected for the second field study (Fig. 1b).

3.3 Efficacy of LLITNs in field conditions

The nets that gave best results in laboratory conditions every year and could be produced on a large scale were tested in field conditions. The nets used had a hole size of 0.46 and 0.29 mm^2 in 2011 and 2013 respectively. In the first field study, the density of alate *A. gossypii* was significantly lower in plots protected by the bifenthrin-treated net than in those covered by the untreated nets from 27 days after aphid release onwards ($U = 443.0$; $Z = -2.3$; $P = 0.02$) (Fig. 2a). Numbers of apterae and nymphs were also lower in plots protected with the bifenthrin-treated nets from 34 days onwards ($U = 390.5$; $Z = -2.7$; $P = 0.07$) and 20 days onwards ($U = 448.0$; $Z = -2.2$; $P = 0.03$) respectively. Plots protected by bifenthrin-treated nets had a significantly lower aphid occupancy rate from the second sampling date onwards ($\chi^2 = 8.52$; $P < 0.01$).

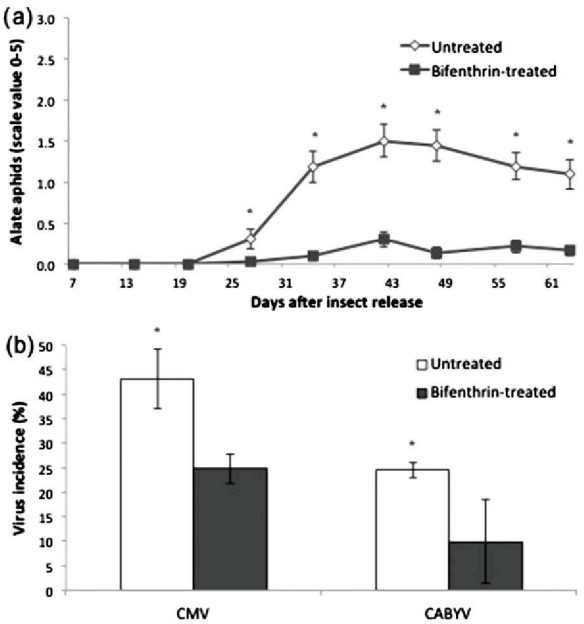

Figure 2. Mean ± SE values: (a) *Aphis gossypii* alate density (scale 0–5) and (b) CMV and CABYV virus transmission (%) inside the plots under bifenthrin-treated and untreated nets during the field experiment in 2011. Asterisks indicate statistical differences according to (a) a one-way ANOVA test followed by DMS ($P < 0.05$) and (b) a chi-square 2 × 2 contingency table goodness-of-fit test ($P < 0.05$).

Furthermore, the incidence of CMV ($\chi^2 = 8.26$; $P < 0.01$) and CABYV ($\chi^2 = 8.07$; $P < 0.01$) was significantly higher in untreated plots than in plots covered by bifenthrin-treated nets (Fig. 2b). Mixed infections with both viruses were also significantly lower in plots

protected with treated nets ($\chi^2 = 7.14$; $P < 0.01$); just one of the three plots had plants infected by both viruses, while the incidence of mixed infection in untreated plots was $13.3 \pm 1.4\%$. However, the net mesh was not dense enough to control whiteflies, and dispersion in plots protected with bifenthrin-treated nets was similar to that under the untreated nets (data not shown). The bifenthrin concentration of the net exposed for 2 months in field conditions during autumn decreased from 3.8 to 3.1 g kg^{-1} net.

According to the results of this first field study, where whiteflies were not effectively excluded, we conducted another field study in 2013 using a net with a smaller pore size (0.29 mm^2), which had promising results under laboratory conditions (Fig. 2b). Results in 2013 followed the same trend as in 2011, with a good control of aphid occupancy from 15 days after aphid release onwards ($\chi^2 = 9.08$; $P < 0.01$) but similar whitefly occupancy in plots protected with the treated and untreated nets ($\chi^2 = 0.51$; $P < 0.51$). Aphids readily entered the control plots 9 days after insect release. However, the bifenthrin-treated net prevented aphid entry for 3 weeks. Virus incidence was not as high as in 2011, but CABYV infection significantly increased inside the untreated plots ($\chi^2 = 8.73$; $P < 0.01$). The bifenthrin concentration was lowered to 1.3 instead of the initial 2.1 g kg^{-1} net at the beginning of the field experiment.

Owing to higher virus transmission, year 2011 data were used to study the spatial distribution of viruses CMV and CABYV and the vector *A. gossypii* in plots with the bifenthrin-treated and untreated nets. Spatial patterns of aphid presence in untreated plots revealed that aphids colonised the entire area of the experimental plot in an aggregated distribution, although this aggregation was not significant (Fig. 3). On the other hand, aphid dispersal was limited to the borders next to insect release in bifenthrin-treated plots, except for the third plot, in which aphid distribution was more uniform. The spread of CMV followed either a random or a regular distribution in the control plots, being significantly regular in the second net-house ($Ia = 0.81$; $P = 0.97$), whereas it was aggregated in the plots protected by bifenthrin-treated nets, with significant aggregation in the second net-house ($Ia = 1.78$; $P = 0.00$) (Fig. 3). The combination of aphid infestation and virus infection showed a significant association between *A. gossypii* and CMV in the third untreated plot ($X = 0.35$; $P = 0.03$) (Fig. 3). For CABYV, the contoured maps of untreated plots showed virus-significant patches restricted to the first two rows of plants that aphids encountered after crossing the untreated net (net-house 1: $Ia = 1.31$; $P = 0.04$; net-house 2: $Ia = 1.57$; $P = 0.00$) (Fig. 4). In contrast, only a few CABYV spots were found in bifenthrin-treated plots, and

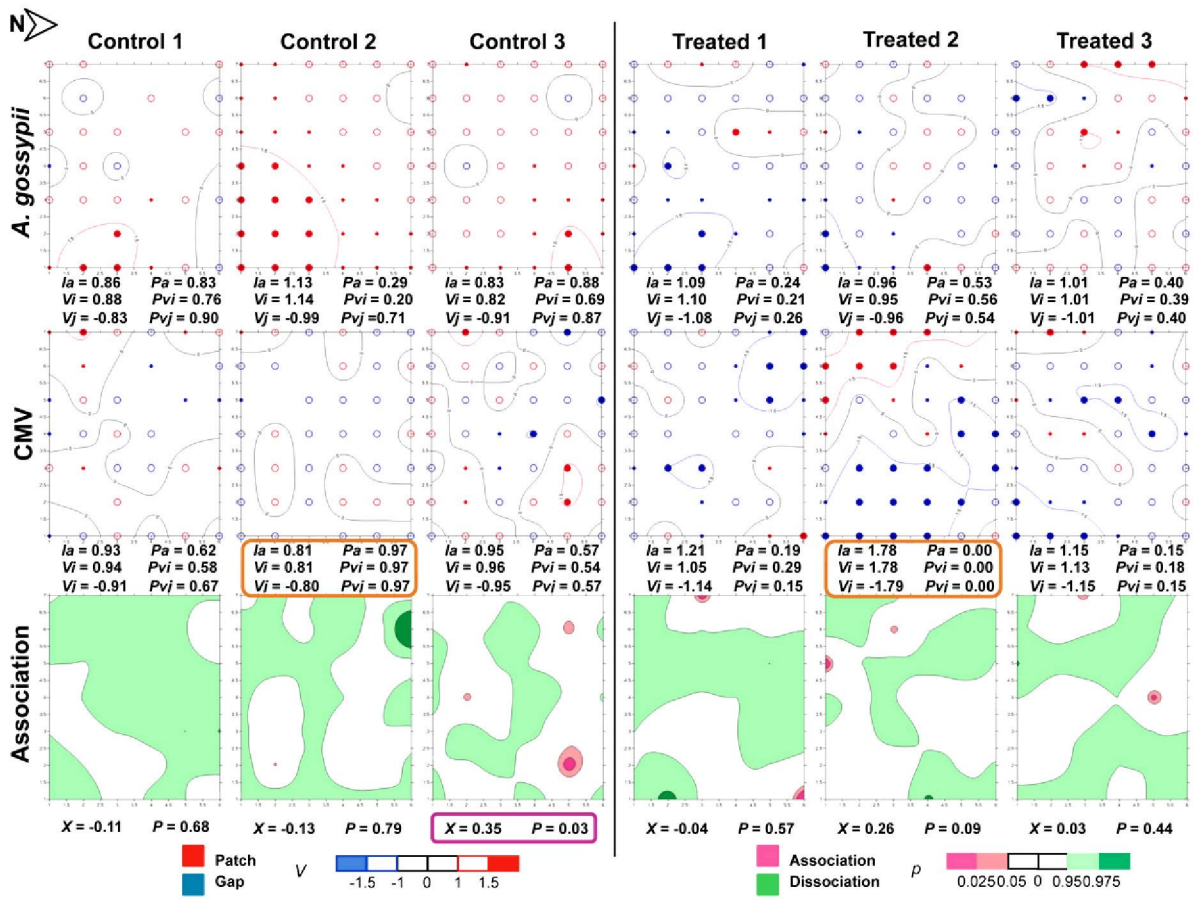

Figure 3. Classed post maps of the spatial distribution of *Aphis gossypii* and CMV-infected plants, and contoured map of the association between CMV-infected plants and its vector, *A. gossypii*, during the field experiment in 2011. Spots indicate individual test plants. Small filled spots represent clustering indices of 0 to ± 0.99 (clustering below expectation), unfilled spots ± 1 to ± 1.49 (clustering slightly exceeding expectation) and large filled spots >1.5 or < 1.5 (more than half as much as expectation). Red lines enclosing patch clusters are contours of $v = 1.5$ and blue lines are contours of $v = -1.5$. Black lines are zero-value contours, representing boundaries between patch and gap regions. The index of aggregation, *Ia*, the positive patch cluster index, *vi*, the negative gap cluster index, *vj*, and the index of spatial association, *X*, circled by coloured lines are statistically significant. The letter N and arrow indicate north orientation.

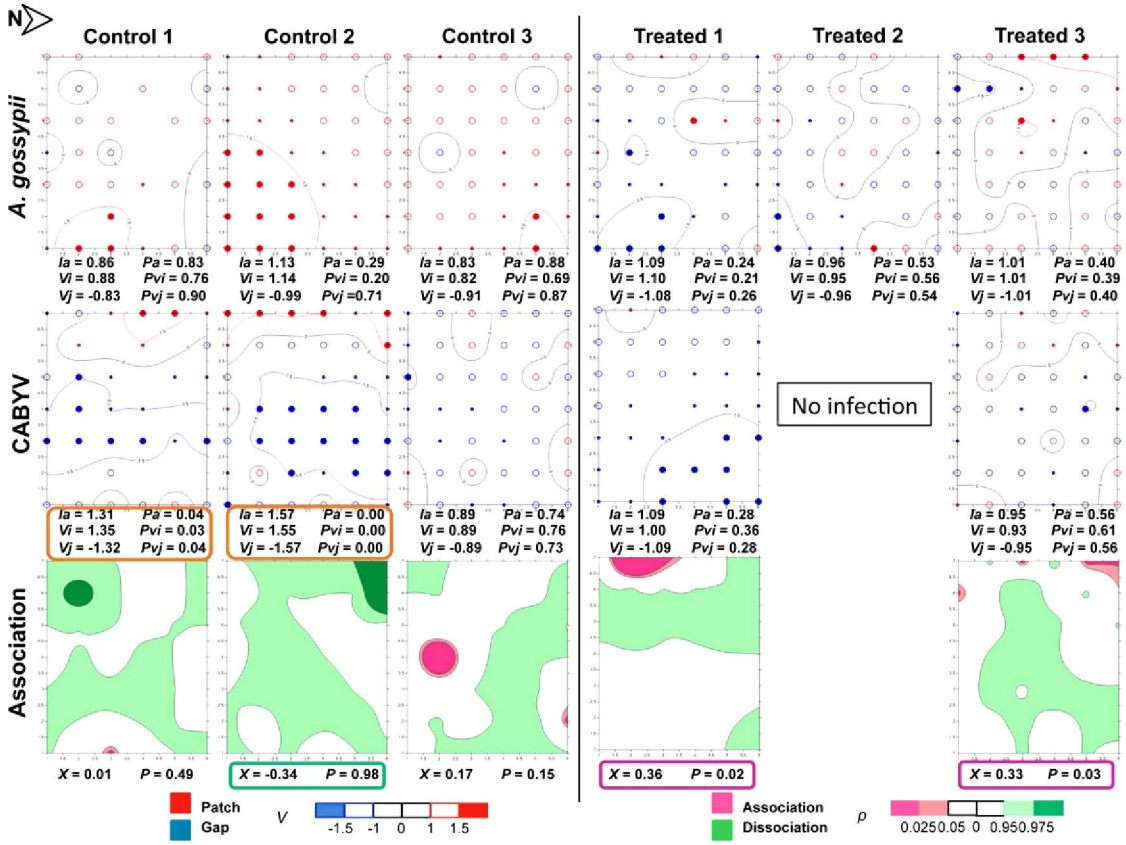

Figure 4. Classed post maps of the spatial distribution of *Aphis gossypii* and CABYV-infected plants, and contoured map of the association between CABYV-infected plants and its vector, *A. gossypii*, during the field experiment in 2011. Symbols and contours are as for Fig. 3.

infection was not detected in the second plot. A significant dissociation between the virus and its vector was recorded in untreated net-house 2 ($X = -0.34$; $P = 0.98$), but a significant aggregation in the border of the treated areas (net-house 1: $X = 0.36$; $P = 0.02$; net-house 3: $X = 0.33$; $P = 0.03$) (Fig. 4).

3.4 Effect of bifenthrin-treated nets on the aphid parasitoid *Aphidius colemani*

Mummies appeared 2 weeks after parasitoid infestation (week 4), and occupancy rate of plants remained constant throughout the crop cycle. The parasitism rate, expressed as number of mummies per *A. gossypii* individuals, was the same in the two treatments on the three parasitoid sampling dates (week 4: untreated 0.37 ± 0.03 versus bifenthrin-treated 0.30 ± 0.14; $t = 0.43$; $P = 0.69$; week 5: untreated 0.91 ± 0.39 versus bifenthrin-treated 0.65 ± 0.46; $t = 0.43$; $P = 0.69$; week 6: untreated 0.92 ± 0.62 versus bifenthrin-treated 0.99 ± 0.88; $t = 0.09$; $P = 0.94$). The average numbers of mummies were statistically similar in untreated (28.33 ± 8.28) and bifenthrin-treated (43.43 ± 8.07) nets ($t = -1.30$; $P = 0.26$).

4 DISCUSSION

The results obtained in this study suggest that LLITNs can be considered as a promising approach for reducing aphid immigration into protected crops while allowing suitable airflow in enclosed environments.[3,4] In addition, these nets produced no harmful effects on *A. colemani*, an aphid parasitoid frequently used as a biocontrol agent in greenhouse production. To our knowledge, this is the first report of the impact of an LLITN on a natural enemy. The size of the net hole was big enough to allow proper ventilation,[4] and at the same time pests that passed through were likely to acquire sufficient pesticide so that the number of living insect pests entering the greenhouse was strongly reduced. In laboratory trials, *M. persicae* and *A. gossypii* access was reduced below 20% in all of the LLITNs tested, even when a low insecticide dosage (2.0 g kg^{-1} net) and large hole size (0.83 mm^2) were used. No differences were observed among the nets studied when they were treated with different doses of deltamethrin. Approximately half of the released aphids were able to reach the target and feed on the leaf when an untreated net of the same mesh as the insecticide-treated net was used as a barrier. Therefore, the incorporation of insecticide to the yarns acted as a chemical barrier against aphids and provided additional benefits to the physical exclusion properties of the net.[8–11] The addition of UV-absorbing additives to the yarn was not an obstacle to the efficacy of the net because mortality was not different when compared with a standard LLITN. In contrast, when PBO alone was used to treat the net, it did not cause significant mortality in aphids and failed to increase efficacy when combined with deltamethrin. Within the experimental design used in this study, the insecticide synergist PBO was not shown to enhance efficacy when combined with pyrethroids in LLITNs, although further investigations are warranted. Lastly, net colours yellow and white had no different effect on aphid mortality.

Our results also indicate no effect of deltamethrin degradation in one of the nets exposed in the field. However, the other two LLITNs tested lost efficacy against *M. persicae* when exposed to

spring conditions, possibly owing to higher temperature and radiation than during winter, although one of them also lost efficacy after winter exposure compared with the unexposed control. The bifenthrin concentration decreased to 3.1 g kg^{-1} net instead of the initial 3.8 g kg^{-1} net after being exposed for 2 months in the field, which strongly reduced the efficacy of the nets against *A. gossypii* and *B. tabaci* under laboratory conditions. In our studies, the persistence of the insecticides appeared to be jeopardised from 1 month onwards, suggesting that nets partially lose efficacy after sun exposure. However, the amount of bifenthrin left in the nets was enough to reduce aphid dispersion and virus spread inside treated net-houses during one growing season. This may be due to the lower insect density during the first weeks of the experiment, as the first vectors that crossed the treated net were impregnated and died before reaching the cucumber crop. Our ongoing work focuses on testing further UV-blocking additives and formulations to maintain the efficacy of these nets over a period longer than a single crop-growing period.

In our field trials, *A. gossypii* density was significantly reduced in cucumber plants protected by completely closed bifenthrin-treated net-houses, so it is likely that our results could be extended to other aphid species.[9–11] Because pyrethroids produce a rapid knockdown effect, the application of LLITNs at field scale may reduce the spread of plant viruses transmitted by aphids, such as CMV and CABYV. In particular, bifenthrin has a slower knockdown effect but better chemical stability when compared with some other pyrethroids, an aspect that needs to be taken into account when developing new LLITNs.[5,25] As shown in the spatial analysis of the field experiment, virus incidence of both viruses and mixed infections significantly decreased under the insecticide-treated net. Different patterns for CMV and CABYV spread inside control plots were also found using SADIE. CMV spread had either a regular or a random distribution in untreated plots, a result that matches the typical spread of non-persistent viruses, as opposed to the aggregation found under plots protected by LLITNs.[31] Moreover, the dispersion of *A. gossypii* was greater inside untreated plots. On the other hand, we found significant CABYV aggregation in the borders of untreated plots, which suggests an initial focus that led to infection in adjacent plants.[32] CABYV spread and aphid density were very limited in bifenthrin-treated plots, which may indicate again a low dispersion rate of both agents under LLITNs. In addition, aphid population was associated with CABYV infected-plants in treated plots, as frequently observed in viruses transmitted in a persistent circulative manner.[32]

Protection against aphids would not be enough if untreated nets were placed as a physical barrier alone in vent openings. A possible solution to this problem would be to reduce the size of the hole to 0.34–0.40 mm.[2,3] One of the drawbacks of this approach could be the insufficient ventilation inside the enclosed structure.[4] Our results suggest that the use of LLITNs either with deltamethrin or bifenthrin might allow adequate ventilation of greenhouses. LLITNs placed in the sides of greenhouses or in window openings would decrease aphid entry, thereby reducing the need for pesticide treatments and increasing the safety of growers and the environment.[11] The use of LLITNs could also help to maintain crop sanitation in regions where vegetable crops are produced using biological control and IPM programmes, as we have shown that nets of this kind can in some circumstances at least be used in a way that is compatible with beneficial insects such as *A. colemani,* an important biocontrol agent. This biocontrol production scheme has reduced the number of insecticide applications when compared with conventional production, but it has also increased the significance of aphids as pests that cause direct damage to plants in south-eastern Spain.[33] In this sense, LLITNs could reduce the risk of aphid infestation. In addition, owing to the increasing importance of biocontrol in greenhouses, further studies would be necessary to assess the compatibility of both strategies.[34]

Notwithstanding, the hole size used was sufficiently large to allow the passage of small insects such as whiteflies. No significant mortality was observed in most nets tested when *B. tabaci* was evaluated, although nets treated with bifenthrin appeared to exclude whiteflies better than the ones treated with deltamethrin. It was necessary to use a 0.60 mm^2 hole size and 5.0 g bifenthrin kg^{-1} net for reasonable blocking of the access of living whiteflies through the net under laboratory conditions. *B. tabaci* is renowned worldwide as an intractable pest that is difficult to control and one that develops pesticide resistance rapidly.[35] Resistance of this whitefly to pyrethroids is well known, and this species is registered in the Arthropod Resistance Pesticide Database.[14] The small size of *B. tabaci* could explain such unsuccessful results, as the body length and width of the whitefly (0.8–0.95 and 0.5 mm respectively) are much smaller than those of aphids.[36] It is likely that, when it crossed the LLITNs, *B. tabaci* was not sufficiently impregnated with the pesticide to suffer from its knockdown effect. The findings of our field experiments also showed that *B. tabaci* was able successfully to cross the bifenthrin-treated nets and disperse over the cucumber plots at a similar rate to when a non-treated net was used as a physical barrier. Our results also agree with the findings on *A. proletella* survival in cabbage field experiments using deltamethrin-impregnated nets as a fence.[9] Recently, promising results have been obtained with the pyrethroid alpha-cypermethrin against whiteflies.[37] The size of the mesh and the chemical compound appear to be major factors in the production of effective LLITNs, and dicofol-impregnated nets controlled injurious mites in eggplants, even though their size was smaller than that of whiteflies.[8] Further experiments should continue to select the most appropriate mesh size and insecticide to exclude *B. tabaci* effectively, but our most recent finding suggests that a pore size of 0.29 mm^2 might be the best compromise to control this species.

When properly designed, LLITNs represent a good strategy that combines chemical and physical control techniques to allow sustainable management and reduce pesticide treatments in crops. Moreover, LLITNs could be implemented in conjunction with the release of commercially available natural enemies. In this study, using laboratory and net-house experiments, we have confirmed that LLITNs can reduce aphid populations as well as decrease the spread of plant viruses. Different mesh sizes and other insecticides should be further assessed for the effective control of *B. tabaci*. It would be interesting to test this strategy at field scale again under commercial greenhouses to develop an alternative tool for IPM programmes.

ACKNOWLEDGEMENTS

We are grateful to Intelligent Insect Control SAS and Ginegar Plastic Products Ltd for the nets, to Enza Zaden España S.L. for the seeds of *Cucumis sativus* var. 'Marumba', to Antonio J Álvarez and Rocío Oliva for measuring the hole size of the nets and to Pedro Hernaiz for supervising the field experiments. This research was supported by the Spanish Ministry of Science and Technology (grants AGL2010-22196-C02-01 and 02). A Moreno, E Viñuela and A Fereres are members of the Associate unit IVAS (CSIC-UPM):

Control of insect vectors of viruses in horticultural sustainable systems.

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